

HUMAN PROTEINS RESPONSIBLE FOR NEDD8 ACTIVATION AND CONJUGATION

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BACKGROUND OF THE INVENTION

Field of the invention

15 The invention relates to covalent modification of proteins through their conjugation with other proteins. More particularly, the invention relates to the modulation of such conjugation involving the protein NEDD8.

Summary of the related art

20 Covalent modification of proteins through their conjugation with other proteins is an important biological mechanism for regulating protein metabolism and biological activity. Herskho and Ciechanover, *Annu. Rev. Biochem.* 61: 761-807 (1992) discloses conjugation of ubiquitin, one of the most conserved eukaryotic proteins, to other proteins through an enzymatic mechanism, as well as its role in
25 protein degradation. Rock *et al.*, *Cell* 78: 761-771 (1994) discloses that ubiquitination of protein antigens is required for processing of such antigens. Murray, *Cell* 81: 149-152 (1995), teaches that ubiquitination of cyclin is involved in cell cycle regulation. Scheffner *et al.*, *Cell* 75: 495-505 (1993) discloses that ubiquitination of p53 is involved in degradation of this tumor suppressor.

30 The enzymatic pathway for ubiquitination has been reasonably well defined. Jentsch, *Annu. Rev. Genet.* 26: 179-207 (1992) discloses that ubiquitination requires initial activation of a conserved C-terminal glycine residue by the ubiquitin

activating enzyme, E1, through formation of ubiquitin adenylate in an ATP-dependent process which liberates PPi, followed by thiol ester formation at a thiol site in E1 with release of AMP. Ubiquitin is then transferred to a thiol site in ubiquitin conjugating enzyme, E2, through formation of a thiol ester bond.

5 Ubiquitin is then transferred to an epsilon amino group of a lysine residue in the target protein through an amide linkage, usually with the involvement of ubiquitin-protein isopeptide ligase, E3. Hopkin, J. Natl. Inst. Health Res. 2: 36-42 (1997), teaches that target specificity is regulated by the particular combination of E2 and E3 protein, with more than 30 E2 proteins and 10 E3 proteins being known at present.

10 Ubiquitin is not the only protein which is used to modify other proteins through covalent linkage, however. Kamitani *et al.*, J. Biol. Chem. 272: 14001-14004 (1997), discloses that sentrin, a ubiquitin-like protein, appears to be processed similarly to ubiquitin, but has a smaller target protein repertoire than ubiquitin. Okura *et al.*, J. Immunol. 272: 4277-4281 (1996) teaches that sentrin protects cells

15 against anti-FAS and tumor necrosis factor-mediated cell death. Loeb and Haas, J. Biol. Chem. 267: 7806-7813 (1992), discloses that ubiquitin cross-reactive protein (UCRP), which contains two ubiquitin domains, is conjugated to a large number of intracellular proteins. Kumar *et al.*, Biochem. Biophys. Res. Commun. 185: 1155-1161 (1992), discloses another ubiquitin-like protein, called NEDD8, for Neural

20 precursor cell-Expressed Developmentally Down regulated. Kamitani *et al.*, J. Biol. Chem. 272: 28557-28562 (1997), teaches that NEDD8 is predominantly expressed in the nucleus and is conjugated to target proteins through a mechanism analogous to ubiquitination.

These proteins, which covalently modify other cellular proteins, are

25 important components of biological regulatory processes. The nuclear expression pattern and developmental regulation of NEDD8 make it a particularly compelling candidate as an important regulatory molecule. There is a need, therefore to understand the role of NEDD8 in biological regulation. Unfortunately, the lack of understanding about the specific proteins involved in NEDD8 conjugation has

resulted in a lack of effective tools to probe the role of NEDD8. There is, therefore, a need for better tools to utilize in elucidating the role of NEDD8 in biological regulation. Ideally, such tools would allow modulation of the activation and/or conjugation of NEDD8.

BRIEF SUMMARY OF THE INVENTION

The invention provides compositions and methods for detecting and/or modulating the conjugation of NEDD8 and/or its transfer to a target protein, as well
5 as compositions and methods for discovering molecules which are useful in detecting and/or modulating the conjugation of NEDD8 and/or its transfer to a target protein. The present invention arises from the purification and characterization of novel NEDD8 activating and conjugating enzymes.

In a first aspect, the invention provides purified NEDD8-activating protein
10 beta subunit (NAE1-beta). The primary amino acid sequence of a preferred embodiment of such NAE1-beta protein is shown in Figure 1.

In a second aspect, the invention provides NAE1-beta expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid sequences encoding NAE1-beta or nucleic acid sequences specifically homologous or
15 specifically complementary thereto, vectors comprising any such nucleic acid sequences and recombinant expression units which express NAE1-beta or, antisense transcripts or dominant negative mutants thereof.

The purified protein and its structural information provided herein enables the preparation of NAE1-beta-binding molecules (NAE1BBMs). Thus, in a third
20 aspect, the invention provides methods for identifying NAE1BBMs. One preferred method according to this aspect of the invention comprises screening for NAE1BBMs by contacting purified NAE1-beta according to the invention and populations of molecules or mixed populations of molecules and determining the presence of molecules which bind specifically to NAE1-beta. Another preferred
25 method according to this aspect of the invention comprises rationally designing molecules to bind NAE1-beta based upon structural information from the purified NAE1-beta protein provided by the invention and determining whether such rationally designed molecules bind specifically to NAE1-beta. This aspect of the invention includes NAE1BBMs identified by the methods according to the

invention.

NAE1BBMs can be used in conventional assays to detect the presence or absence, and/or quantity of NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Thus, in a fourth aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Such methods comprise providing a detectable NAE1BBM to a biological sample, allowing the detectable NAE1BBM to bind to NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NAE1BBM and NAE1-beta, NAE1-heterodimer, or NAE1 heterodimer/NEDD8 complex.

Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NAE1-beta can also be used in conventional assays to detect the presence or absence of NAE1-beta nucleic acid in a biological sample. Thus, in a fifth aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-beta nucleic acid in a biological sample. In preferred embodiments, such assays are nucleic acid hybridization and/or amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically complementary to NAE1-beta nucleic acid.

In a sixth aspect, the invention provides methods for identifying modulating ligands of NAE1-beta. Some NAE1BBMs are capable of acting as antagonists or agonists of NAE1-beta. Thus, the method according to this aspect of the invention comprises providing NAE1BBMs to an assay system for NAE1-beta participation in the NEDD8-activation/conjugation pathway, and determining whether such NAE1BBMs interfere with or enhance the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. The NAE1BBMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a

mixed population of molecules, as for example in a screening procedure. This aspect of the invention includes modulating ligands of NAE1-beta identified by this method according to the invention.

5 In a seventh aspect, the invention provides modulating ligands of NAE1-beta. Preferred modulating ligands are NAE1BBMs which act as antagonists, interfering with the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are NAE1BBMs which act as agonists, enhancing the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. In certain embodiments, such
10 NAE1BBMs preferably interact with NAE1-beta to inhibit or enhance the formation of NAE1 heterodimer, the formation of NEDD8 adenylate, the formation of a thiol ester bond between NEDD8 and NAE1, and/or transfer of NEDD8 to NEDD8-conjugating enzyme.

15 In an eighth aspect, the invention provides methods for modulating the activation and/or conjugation of NEDD8. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-beta or a recombinant expression unit which expresses NAE1-beta or an antagonist thereof to a biological system in which NEDD8 is conjugated to another protein.

20 In a ninth aspect, the invention provides oligonucleotides that are specifically complementary to a portion of a nucleotide sequence shown in Figure 1. Preferred embodiments include hybridization probes and antisense oligonucleotides.

25 In a tenth aspect, the invention provides methods for identifying NAE1-alpha binding molecules (NAE1ABMs). The present inventors have identified the alpha subunit of the NAE1 heterodimer (NAE1-alpha). Surprisingly, it has an amino acid sequence which is substantially identical to a protein previously identified as amyloid precursor protein binding protein 1 (APP-BP1; see Chow *et al.*, J. Biol. Chem. 271: 11339-11346 (1996)) One preferred method according to this aspect of the invention comprises screening for NAE1ABMs by contacting purified NAE1-

alpha and populations of molecules or mixed populations of molecules and determining the presence of molecules which bind specifically to NAE1-alpha. Another preferred method according to this aspect of the invention comprises rationally designing molecules to bind NAE1-alpha based upon structural information from the NAE1-alpha protein identified by the present inventors and determining whether such rationally designed molecules bind specifically to NAE1-alpha. This aspect of the invention includes NAE1ABMs identified by the methods according to the invention.

NAE1ABMs can be used in conventional assays to detect the presence or absence, and/or quantity of NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Thus, in an eleventh aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Such methods comprise providing a detectable NAE1ABM to a biological sample, allowing the detectable NAE1ABM to bind to NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NAE1ABM and NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex. In preferred embodiments, the method according to this aspect of the invention is used to detect the presence or absence, and/or quantity of NAE1 heterodimer or NAE1 heterodimer/NEDD8 complex in a biological sample.

Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NAE1-alpha can also be used in conventional assays to detect the presence or absence of NAE1-alpha nucleic acid in a biological sample in which NEDD8 conjugation is suspected. Thus, in a twelfth aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-alpha nucleic acid in such a biological sample. In preferred embodiments, such assays are nucleic acid hybridization and/or

amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically complementary to NAE1-alpha nucleic acid.

5 In an thirteenth aspect, the invention provides methods for identifying modulating ligands of NAE1-alpha. Some NAE1ABMs are capable of acting as antagonists or agonists of NAE1-alpha. Thus, the method according to this aspect of the invention comprises providing NAE1ABMs to an assay system for NAE1-alpha participation in the NEDD8-activation/conjugation pathway, and determining whether such NAE1ABMs interfere with or enhance the ability of NAE1-alpha to
10 participate in the NEDD8-activation/conjugation pathway. The NAE1ABMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a mixed population of molecules, as for example in a screening procedure. This aspect of the invention includes antagonists or agonists of NAE1-alpha identified by this method according to the invention.

15 In a fourteenth aspect the invention provides a purified complex of NAE1-beta and NAE1-alpha, or of NAE1-beta, NAE1-alpha and NEDD8, or a purified complex of portions thereof.

In a fifteenth aspect, the invention provides modulating ligands of NAE1-alpha. Certain preferred modulating ligands are NAE1ABMs which act as
20 antagonists which interfere with the ability of NAE1-alpha to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are NAE1ABMs which act as agonists which enhance the ability of NAE1-alpha to participate in the NEDD8-activation/conjugation pathway. Preferably, such inhibition or enhancement is specific, as described above. In certain embodiments,
25 such modulating ligands preferably interact with NAE1-alpha to inhibit or enhance the formation of NAE1 heterodimer, the formation of NEDD8 adenylate, the formation of a thiol ester bond between NEDD8 and NAE1, and/or transfer of NEDD8 to NEDD8-conjugating enzyme.

In a sixteenth aspect, the invention provides methods for modulating the

activation and/or conjugation of NEDD8. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand NAE1-alpha or a recombinant expression unit which expresses NAE1-alpha or an antagonist thereof to a biological system in which NEDD8 is conjugated to another protein.

In a seventeenth aspect, the invention provides allelic variants of NAE-1 alpha. This aspect of the invention further includes NAE1-alpha allelic variant expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid sequences encoding NAE1-alpha, or nucleic acid sequences specifically homologous or specifically complementary thereto, vectors comprising any such nucleic acid sequences, and recombinant expression units which express NAE1-beta or antisense transcripts or dominant negative mutants thereof.

In a eighteenth aspect, the invention provides methods for modulating auxin response in plants. The present inventors have discovered that NAE1-alpha shares 39% identity and 61% conserved residues with Aux1 in *A. Thaliana*, which is involved in signal transduction in the auxin response in plants. This suggests that antagonists of NAE1-beta and/or NAE1-alpha should down-regulate the auxin response, and that expression of NAE1-beta and/or NAE1-alpha should up-regulate the auxin response. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-beta or NAE1-alpha or a recombinant expression unit which expresses NAE1-beta or NAE1 or an antagonist thereof to a plant that is under auxin treatment.

In a nineteenth aspect, the invention provides methods for modulating the biological role of APP and/or beta peptide accumulation in a biological system. The present inventors have discovered that NAE1-alpha is substantially the same protein as amyloid precursor protein binding protein-1 (APP-BP1). This suggests that antagonists or agonists of NAE1-beta and/or NAE1-alpha should modulate APP function, including its role in beta peptide accumulation. One preferred embodiment of the method according to this aspect of the invention comprises

providing a modulating ligand of NAE1-beta or NAE1-alpha or a recombinant expression unit which expresses NAE1-beta or NAE1 or an antagonist thereof to a biological system.

5 In an twentieth aspect, the invention provides two new purified NEDD8-conjugating enzymes and allelic variants thereof. The primary amino acid sequence of a preferred embodiment of a first such NEDD8-conjugating enzyme (NCE1) is shown in Figure 2. The primary amino acid sequence of a preferred embodiment of a second such NEDD8-conjugating enzyme (NCE2) is shown in Figure 5.

10 In a twenty-first aspect, the invention provides NEDD8-conjugation enzyme expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid sequences encoding NCE1 or NCE2 or dominant negative mutants thereof, or capable of expressing antisense transcripts thereof or nucleic acid sequences specifically homologous or specifically complementary thereto, and vectors comprising any such recombinant expression elements, preferably
15 expression vectors.

The purified protein and its structural information provided herein enables the preparation of NCE1 and NCE2 binding molecules, respectively NCE1BMs and NCE2BMs. Thus, in a twenty-second aspect, the invention provides methods for identifying NCE1BMs and NCE2BMs. One preferred method according to this aspect
20 of the invention comprises screening for NCE1BMs or NCE2BMs by contacting purified NCE1 or NCE2 according to the invention and populations of molecules or mixed populations of molecules and determining the presence of molecules which bind specifically to NCE1 or NCE2. Another preferred method according to this aspect of the invention comprises rationally designing molecules to bind NCE1 or
25 NCE2 based upon structural information from the purified NCE1 or NCE2 provided by the invention and determining whether such rationally designed molecules bind specifically to NCE1 or NCE2. This aspect of the invention includes NCE1BMs and NCE2BMs identified by the methods according to the invention.

NCE1BMs and NCE2BMs can be used in conventional assays to detect the

presence or absence, and/or quantity of NCE1 or NCE2, or NCE1 or NCE2/NEDD8 complex in a biological sample. Thus, in a twenty-third aspect, the invention provides methods for determining the presence or absence and/or quantity of NCE1 or NCE2, or NCE1 or NCE2/NEDD8 complex in a biological sample. Such methods
5 comprise providing a detectable NCE1BM or NCE2BM to a biological sample, allowing the detectable NCE1BM or NCE2BM to bind to, respectively NCE1 or NCE2, or respectively NCE1 or NCE2/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NCE1BM or NCE2BM and NCE1 or NCE2, or NCE1 or NCE2/NEDD8
10 complex.

Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NCE1 or NCE2 can also be used in conventional assays to detect the presence or absence of NCE1 or NCE2 nucleic acid
15 in a biological sample. Thus, in a twenty-fourth aspect, the invention provides methods for determining the presence or absence and/or quantity of NCE1 or NCE2 nucleic acid in a biological sample. In preferred embodiments, such assays are nucleic acid hybridization and/or amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically
20 complementary to NCE1 or NCE2 nucleic acid.

In a twenty-fifth aspect, the invention provides methods for identifying
25 modulating ligands of NCE1 or NCE2. Some NCE1BMs or NCE2BMs are capable of acting as antagonists or agonists of, respectively NCE1 or NCE2. Thus, the method according to this aspect of the invention comprises providing NCE1BMs or NCE2BMs to an assay system for NCE1 or NCE2 participation in the NEDD8-
30 activation/conjugation pathway, and determining whether such NCE1BMs or NCE2BMs interfere with or enhance the ability of NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. The NCE1BMs or NCE2BMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a mixed population of molecules, as for example in a

screening procedure. This aspect of the invention includes modulating ligands of NCE1 or NCE2 identified by this method according to the invention.

5 In a twenty-sixth aspect, the invention provides modulating ligands of NCE1 or NCE2. Preferred modulating ligands are NCE1BMs or NCE2BMs which act as antagonists, interfering with the ability of NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are NCE1BMs or NCE2BMs which act as agonists, enhancing the ability of, respectively NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. In
10 certain embodiments, such NCE1BMs or NCE2BMs preferably interact with NCE1 or NCE2 to inhibit or enhance the formation of a thiol ester bond between NEDD8 and NCE1 or NCE2 and/or transfer of NEDD8 to its target protein.

In a twenty-seventh aspect, the invention provides methods for modulating the conjugation of NEDD8 or its transfer to a target protein. One preferred
15 embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NCE1 or NCE2 or a recombinant expression unit which expresses NCE1 or NCE2 or an antagonist thereof to a biological system in which NEDD8 is conjugated to another protein.

In a twenty-eighth aspect, the invention provides oligonucleotides that are specifically complementary to a portion of a nucleotide sequence shown in Figure 2
20 or Figure 5. Preferred embodiments include hybridization probes and antisense oligonucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows the nucleotide [SEQ. ID. NO. 1] and predicted amino acid sequence [SEQ. ID. NO. 2] for NAE1-beta, with the two tryptic peptide sequences highlighted by underline.

 Figure 2 shows the nucleotide [SEQ. ID. NO. 3] and predicted amino acid sequence [SEQ. ID. NO. 4] for NEDD8-conjugating enzyme 1 (NCE1), with the active Cys residue indicated.

10 Figure 3 shows the alignment of NCE1 with yeast Ubc12.

 Figure 4 shows results of an assay for thioester bond formation between NEDD-8 and NCE1.

15 Figure 5 shows the nucleotide [SEQ. ID. NO. 5] and predicted amino acid sequence [SEQ. ID. NO. 6] for NEDD8-conjugating enzyme 2 (NCE2), with the active Cys residue indicated.

 Figure 6 shows homology between NCE2 and a *C. elegans* gene of unknown function.

 Figure 7 shows the sequence alignment of NCE1 and NCE2 with known Ubc proteins.

20 Figure 8 shows results of an assay for thioester bond formation between NEDD8 and NCE2.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The invention relates to covalent modification of proteins through their conjugation with other proteins. More particularly, the invention relates to the modulation of such conjugation involving the protein NEDD8. The invention provides compositions and methods for detecting and/or modulating the conjugation of NEDD8 and/or its transfer to a target protein, as well as compositions and methods for discovering molecules which are useful in detecting and/or modulating the conjugation of NEDD8 and/or its transfer to a target protein. The present invention arises from the purification and characterization of novel NEDD8 activating and conjugating enzymes.

10 The patents and publications cited herein reflect the knowledge in the art and are hereby incorporated by reference in entirety. Any inconsistency between these patents and publications and the present disclosure shall be resolved in favor of the present disclosure.

15 In a first aspect, the invention provides purified NEDD8-activating protein beta subunit (NAE1-beta). The primary amino acid sequence of a preferred embodiment of such NAE1-beta protein is shown in Figure 1. However, the term "NEDD8-activating protein beta subunit", or "NAE1-beta", is intended to include allelic variants thereof. An "allelic variant", as used herein, is a protein having at least about 75% amino acid sequence, preferably at least about 85%, more preferably at least about 95%, and most preferably at least about 99% identity to the amino acid sequence set forth in SEQ ID NO ~~2~~¹, or to a portion or protein conjugate thereof which retains the biological activity of NAE1-beta (as part of the NAE1 heterodimer) to form a thioester linkage with NEDD8 at a rate faster than that achieved by human ubiquitin activating enzyme 1, preferably at least 2-fold faster, more preferably at least 5-fold and most preferably at least 10-fold. Alternatively, an allelic variant can retain such biological activity and comprise a peptide sequence having at least 70%

amino acid identity to the peptide sequence corresponding to residues 46-118 in Figure 1, or at least 45% amino acid identity to the peptide sequence corresponding to residues 119-166, at least 55% amino acid identity to the peptide sequence corresponding to residues 175-239, or at least 35% amino acid identity to the peptide sequence corresponding to residues 276-375. Preferably such biologically active portion comprises at least the PXCT motif, wherein X can be any amino acid, preferably a hydrophobic amino acid, more preferably methionine, leucine, or isoleucine, and most preferably methionine. More preferably, such biologically active portion comprises amino acid sequence of residues 214-217, more preferably comprises at least about 25 additional amino acids of NAE1-beta, even more preferably at least about 50 additional amino acids of NAE1-beta, still more preferably at least about 75 additional amino acids of NAE1-beta, yet even more preferably at least about 100 additional amino acids of NAE1-beta, most preferably at least about 150 additional amino acids from NAE1-beta. Such allelic variants have the biological activity of NAE1-beta, as discussed above, which is the catalytic monomer of the NAE1 heterodimer. In alternative preferred embodiments, such allelic variants are either rationally designed or naturally occurring allelic variants, *i.e.*, they are expressed in actual individual mammals, most preferably from actual individual humans or mice. Rationally designed allelic variants can be produced according to standard art-recognized procedures (see *e.g.*, international publication WO95/18974). "Purified", as used herein means having less than about 25% by weight, and preferably less than about 10% by weight contamination with other proteins. Such purified proteins may be obtained from natural sources, from recombinant expression, or by chemical synthesis. "Protein", as used herein and hereinbelow is intended to encompass any polypeptide having at least 10 amino acid residues.

In a second aspect, the invention provides NAE1-beta expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid

sequences encoding NAE1-beta or dominant negative mutants thereof, or capable of expressing antisense transcripts thereof or nucleic acid sequences specifically homologous or specifically complementary thereto, and vectors comprising any such recombinant expression elements, preferably expression vectors.

- 5 For purposes of the invention, amino acid sequence identity and homology are determined using the program Clustal W Version 1.6 to do sequence alignment (Thompson *et al.*, Nucleic Acids Res 22: 4673-4680 (1994)). For viewing aligned sequences, the program GeneDoc Version 2.2 was used. A sequence is "specifically homologous" to another sequence if it is sufficiently homologous to specifically
- 10 hybridize to the exact complement of the sequence. A sequence is "specifically complementary" to another sequence if it is sufficiently homologous to specifically hybridize to the sequence. A sequence "specifically hybridizes" to another sequence if it hybridizes to form Watson-Crick or Hoogsteen base pairs either in the body, or under conditions which approximate physiological conditions with respect to ionic
- 15 strength, *e.g.*, 140 mM NaCl, 5 mM MgCl₂. Preferably, such specific hybridization is maintained under stringent conditions, *e.g.*, 0.2X SSC at 68°C. A "recombinant expression element" is a nucleic acid sequence which encodes NAE1-beta, or a portion encoding at least 15 contiguous amino acids thereof, or a dominant negative mutant thereof, or is capable of expressing an antisense molecule specifically
- 20 complementary thereto, or a sense molecule specifically homologous thereto wherein the recombinant expression unit may be in the form of linear DNA or RNA, covalently closed circular DNA or RNA, or as part of a chromosome, provided however that it cannot be the native chromosomal locus for NAE1-beta. Preferred recombinant expression elements are vectors, which may include an
- 25 origin of replication and are thus replicatable in one or more cell type. Certain preferred recombinant expression elements are expression vectors, and further comprise at least a promoter and passive terminator, thereby allowing transcription of the recombinant expression element in a bacterial, fungal, plant, insect or mammalian cell. Preferred recombinant expression elements have at least 75%

nucleic acid sequence identity with the nucleic acid sequence set forth in SEQ ID NO 1, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 99%, and encode a protein or peptide having either NAE1-beta biological activity, as described above, or activity as a dominant negative mutant thereof, as further described below.

“Dominant negative mutants” are proteins derived from NAE1-beta or NAE1-alpha which inhibit the biological activity of NAE1. Preferred dominant negative mutants include allelic variants in which the C at position 216 is substituted, preferably by S. Additional preferred dominant negative mutants interfere with association of native NAE1-beta with native NAE1-alpha and can be derived from either NAE1-beta and NAE1-alpha. Such dominant negative mutants can be prepared by art recognized procedures (see *e.g.*, Townsley *et al.*, Proc. Natl. Acad. Sci. USA 94: 2362-2367 (1997)). Preferably, such dominant negative mutant is a protein or peptide having from 50% amino acid sequence identity to about 99% identity to the amino acid sequence set forth in SEQ ID NO 2, or to a portion or protein conjugate thereof which inhibits the biological activity of NAE1 to form a thioester linkage with NEDD8 or transfer NEDD8 to a NEDD8 conjugating enzyme, under conditions as described in the following examples by at least 50%, preferably by at least 75%, more preferably by at least 90% and most preferably by at least 99%.

Preferably, such inhibitory portion comprises an amino acid sequence spanning residue 216, more preferably comprises at least about 25 additional amino acids of NAE1-beta, or at least about 50 additional amino acids of NAE1-beta, or at least about 75 additional amino acids of NAE1-beta, or at least about 100 additional amino acids of NAE1-beta, or even at least about 150 additional amino acids of NAE1-beta. For purposes of this aspect of the invention, the term “spanning residue 216” means comprising amino acid residues in both the N-terminal and C-terminal directions from residue 216, as that residue is shown in Figure 1. Preferably, residue 216 itself may be substituted by one or more amino acids, more preferably from about 1 to about 50 amino acids, or residue 216 may be absent. Preferably the amino acids in

the N-terminal and C-terminal directions from residue 216 are each independently within 20 amino acids of residue 216, as shown in Figure 1, more preferably within 10, even more preferably within 5, and most preferably are immediately adjacent residue 216 as shown in Figure 1.

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The purified protein and its structural information provided herein enables the preparation of NAE1-beta-binding molecules (NAE1BBMs). Thus, in a third aspect, the invention provides methods for identifying NAE1BBMs. One preferred method according to this aspect of the invention comprises screening for
10 NAE1BBMs by contacting purified NAE1-beta according to the invention and populations of molecules or mixed populations of molecules and determining the presence of molecules which bind specifically to NAE1-beta. Another preferred method according to this aspect of the invention comprises rationally designing
15 NAE1-beta protein and amino acid sequence disclosed herein provided by the invention and determining whether such rationally designed molecules bind specifically to NAE1-beta. Molecules that bind specifically to NAE1-beta are molecules that bind to NAE1-beta with greater affinity than to other unrelated
20 proteins. Preferably, binding affinity of the molecule is at least 5-fold greater than affinity for unrelated proteins, more preferably at least 10-fold greater, still more preferably at least 50-fold greater, and most preferably at least 100-fold greater. This aspect of the invention includes NAE1BBMs identified by the methods according to the invention.

As used herein, a "NAE1-beta-binding molecule", or "NAE1BBM", is a
25 molecule or macromolecule which binds under physiological conditions to NAE1-beta. "Binds under physiological conditions" means forming a covalent or non-covalent association with an affinity of at least 10^6 M^{-1} , most preferably at least 10^9 M^{-1} , either in the body, or under conditions which approximate physiological conditions with respect to ionic strength, e.g., 140 mM NaCl, 5 mM MgCl_2 . A

"population of molecules", as used herein, refers to a plurality of identical molecules. A "mixed population of molecules" refers to a plurality of molecules wherein more than one type of molecule is present.

5 In certain preferred embodiments, an NAE1BBM according to the invention is a peptide or a peptidomimetic. For purposes of the invention, a "peptide" is a molecule comprised of a linear array of amino acid residues connected to each other in the linear array by peptide bonds. Such peptides according to the invention may include from about three to about 500 amino acids, and may further include secondary, tertiary or quaternary structures, as well as intermolecular associations
10 with other peptides or other non-peptide molecules. Such intermolecular associations may be through, without limitation, covalent bonding (e.g., through disulfide linkages), or through chelation, electrostatic interactions, hydrophobic interactions, hydrogen bonding, ion-dipole interactions, dipole-dipole interactions, or any combination of the above.

15 In certain preferred embodiments, such an NAE1BBM comprises a complementarity determining region of an antibody which binds under physiological conditions to a peptide-containing epitope of NAE1-beta, or a peptidomimetic of such a complementarity determining region. For purposes of the invention, a "complementarity determining region of an antibody" is that portion
20 of an antibody which binds under physiological conditions to an epitope, including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the human heavy chain V, D and J regions, the human light chain V and J regions, and/or combinations thereof. Examples of such preferred embodiments include an antibody, or an
25 antibody derivative, which may more preferably be a monoclonal antibody, a human antibody, a humanized antibody, a single-chain antibody, a chimeric antibody, or an antigen-binding antibody fragment.

Those skilled in the art are enabled to make any such antibody derivatives using standard art-recognized techniques. For example, Jones *et al.*, Nature 321:

522-525 (1986) discloses replacing the CDRs of a human antibody with those from a mouse antibody. Marx, Science 229: 455- 456 (1985) discusses chimeric antibodies having mouse variable regions and human constant regions. Rodwell, Nature 342: 99-100 (1989) discusses lower molecular weight recognition elements derived from antibody CDR information. Clackson, Br. J. Rheumatol. 3052: 36-39 (1991) discusses genetically engineered monoclonal antibodies, including Fv fragment derivatives, single chain antibodies, fusion proteins chimeric antibodies and humanized rodent antibodies. Reichman *et al.*, Nature 332: 323-327 (1988) discloses a human antibody on which rat hypervariable regions have been grafted. Verhoeyen, *et al.*, Science 239: 1534-1536 (1988) teaches grafting of a mouse antigen binding site onto a human antibody.

In addition, those skilled in the art are enabled to design and produce peptidomimetics having binding characteristics similar or superior to such complementarity determining region (see *e.g.*, Horwell *et al.*, Bioorg. Med. Chem. 4: 1573 (1996); Liskamp *et al.*, Recl. Trav. Chim. Pays- Bas 1: 113 (1994); Gante *et al.*, Angew. Chem. Int. Ed. Engl. 33: 1699 (1994); Seebach *et al.*, Helv. Chim. Acta 79: 913 (1996)). Accordingly, all such antibody derivatives and peptidomimetics thereof are contemplated to be within the scope of the present invention. Compositions according to the invention may further include physiologically acceptable diluents, stabilizing agents, localizing agents or buffers.

Additional preferred NAE1BBMs according to the invention include small molecules, which can be identified using screening or rational design approaches as discussed later herein.

NAE1BBMs can be used in conventional assays to detect the presence or absence, and/or quantity of NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Thus, in a fourth aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex

in a biological sample. Such methods comprise providing a detectable NAE1BBM to a biological sample, allowing the detectable NAE1BBM to bind to NAE1-beta, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NAE1BBM and NAE1-beta, NAE1-heterodimer, or NAE1 heterodimer/NEDD8 complex.

A detectable NAE1BBM is an NAE1BBM which can be detected in an assay. Such detection is preferably through the direct or indirect binding of a tag or label on the NAE1BBM. "Direct or indirect binding" means that the tag or label may be directly connected to the NAE1BBM by intermolecular association, or may be connected via intermediate molecules to the NAE1BBM by intermolecular association. Such intermolecular associations may be through, without limitation, covalent bonding (*e.g.*, through disulfide linkages), or through chelation, electrostatic interactions, hydrophobic interactions, hydrogen bonding, ion-dipole interactions, dipole-dipole interactions, or any combination of the above. Preferred tags and labels include, without limitation, radioisotopes, heavy metals, fluorescent labels, chemoluminescent labels, enzymes and enzyme substrates. Preferred biological samples include blood, serum, plasma, cells, tissue portions, and cell or tissue extracts. In certain preferred embodiments, the method according to this aspect of the invention takes the form of a conventional ELISA or RIA. In another preferred embodiment, the method employs either direct or indirect immunofluorescence. Additional preferred embodiments utilize *in vivo* imaging of cells expressing NAE1-beta using conventional imaging agents directly or indirectly bound to an NAE1BBM according to the invention.

Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NAE1-beta can also be used in conventional assays to detect the presence or absence of NAE1-beta nucleic acid in a biological sample. Thus, in a fifth aspect, the invention provides methods for

determining the presence or absence and/or quantity of NAE1-beta nucleic acid in a biological sample. In preferred embodiments, such assays are nucleic acid hybridization and/or amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically complementary to NAE1-beta nucleic acid. Particularly preferred embodiments include Northern blotting, dot or slot blotting, and polymerase chain reaction.

In a sixth aspect, the invention provides methods for identifying modulating ligands of NAE1-beta. Some NAE1BBMs are capable of acting as antagonists or agonists of NAE1-beta. Thus, the method according to this aspect of the invention comprises providing NAE1BBMs to an assay system for NAE1-beta participation in the NEDD8-activation/conjugation pathway, and determining whether such NAE1BBMs interfere with or enhance the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. The NAE1BBMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a mixed population of molecules, as for example in a screening procedure. This aspect of the invention includes antagonists or agonists of NAE1-beta identified by this method according to the invention. Assessment of ability to "interfere with or enhance the ability to participate in the NEDD8-activation/conjugation pathway" can conveniently be carried out using an *in vitro* activity system, as later described herein. Preferably, such interference or enhancement results in a reduction of NEDD8 activation/conjugation of at least 50%, more preferably at least 90%, and most preferably, at least 99%, or an increase of NEDD8 activation/conjugation of at least 50%, preferably at least 2-fold, more preferably at least 5-fold.

In a seventh aspect, the invention provides modulating ligands of NAE1-beta. Preferred modulating ligands are NAE1BBMs which act as antagonists, interfering with the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are

NAE1BBMs which act as agonists, enhancing the ability of NAE1-beta to participate in the NEDD8-activation/conjugation pathway. Preferably, such inhibition or enhancement is specific, *i.e.*, the modulating ligand interferes with or enhances the ability of NAE1-beta to participate in the NEDD8 activation/ conjugation pathway at a concentration that is lower than the concentration of the ligand required to produce another, unrelated biological effect. Preferably, the concentration of the ligand required for NEDD8 activation/conjugation modulating activity is at least 2-fold lower, more preferably at least 5-fold lower, even more preferably at least 10-fold lower, and most preferably at least 20-fold lower than the concentration required to produce an unrelated biological effect. In certain embodiments, such NAE1BBMs preferably interact with NAE1-beta to inhibit or enhance the formation of NAE1 heterodimer, the formation of NEDD8 adenylate, the formation of a thiol ester bond between NEDD8 and NAE1, and/or transfer of NEDD8 to NEDD8-conjugating enzyme.

In an eighth aspect, the invention provides methods for modulating the conjugation of NEDD8 to NAE1 or its transfer to a NEDD8 conjugating enzyme or a target protein. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-beta or a recombinant expression unit which expresses NAE1-beta or an antagonist thereof to a biological system in which NEDD8 is conjugated to a NEDD8 conjugating enzyme or a target protein.

The term "biological system", as used herein, includes *in vitro* cell or tissue extracts, cell cultures, tissue cultures, organ cultures, living plants and animals, including mammals, including without limitation humans and mice. An "antagonist" is a molecule which inhibits the biological activity of NAE1.

In a ninth aspect, the invention provides oligonucleotides that are specifically complementary to a portion of a nucleotide sequence shown in Figure 1. Preferred

embodiments include hybridization probes and antisense oligonucleotides.

For purposes of the invention, the term oligonucleotide includes polymers of two or more deoxyribonucleotide, or any modified nucleoside, including 2'-halo-nucleosides, 2'-O-substituted ribonucleosides, deazanucleosides or any combination thereof. Preferably, such oligonucleotides have from about 10 to about 100 nucleosides, more preferably from about 15-50, and most preferably from about 15 to 35. Such monomers may be coupled to each other by any of the numerous known internucleoside linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines and adamantane. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with a halogen (preferably Cl, Br, or F), or an O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group. Certain embodiments of such oligonucleotides are useful in hybridization assays. Other embodiments are useful as antisense oligonucleotides for use in animal model or human therapeutic settings.

In a tenth aspect, the invention provides methods for identifying NAE1-alpha binding molecules (NAE1ABMs). The present inventors have identified the alpha subunit of the NAE1 heterodimer (NAE1-alpha). Surprisingly, it has an amino acid sequence which is substantially identical to a protein previously

identified as amyloid precursor protein binding protein 1 (APP-BP1; see Chow *et al.*, J. Biol. Chem. 271: 11339-11346 (1996)) One preferred method according to this aspect of the invention comprises screening for NAE1ABMs by contacting purified NAE1-alpha and populations of molecules or mixed populations of molecules and
5 determining the presence of molecules which bind specifically to NAE1-alpha, or preferably to NAE1 heterodimer. Another preferred method according to this aspect of the invention comprises rationally designing molecules to bind NAE1-alpha based upon structural information from the NAE1-alpha protein identified by the present inventors and determining whether such rationally designed molecules
10 bind specifically to NAE1-alpha. This aspect of the invention includes NAE1ABMs identified by the methods according to the invention.

The terms "bind specifically", "population of molecules" and "mixed population of molecules" are as described previously. Structural aspects of NAE1ABMs are as discussed above for NAE1BBMs, except that NAE1ABMs bind
15 under physiological conditions to NAE1-alpha. Preferably, binding affinity of the molecule for NAE1-alpha is at least 5-fold greater than affinity for unrelated proteins, more preferably at least 10-fold greater, still more preferably at least 50-fold greater, and most preferably at least 100-fold greater. This aspect of the invention includes NAE1ABMs identified by the methods according to the invention.

20 As used herein, a "NAE1-alpha-binding molecule", or "NAE1ABM", is a molecule or macromolecule which binds under physiological conditions to NAE1-alpha. The terms "binds under physiological conditions", "population of molecules", and "mixed population of molecules" are as used previously.

In certain preferred embodiments, an NAE1ABM according to the invention
25 is a peptide or a peptidomimetic. For purposes of the invention, the term "peptide" is as used previously.

In certain preferred embodiments, such an NAE1ABM comprises a complementarity determining region of an antibody which binds under physiological conditions to a peptide-containing epitope of NAE1-alpha, or a

peptidomimetic of such a complementarity determining region. For purposes of the invention, the term "complementarity determining region of an antibody" is as used previously. Compositions according to the invention may further include physiologically acceptable diluents, stabilizing agents, localizing agents or buffers.

5 Additional preferred NAE1ABMs according to the invention include small molecules, which can be identified using screening or rational design approaches as discussed later herein.

10 NAE1ABMs can be used in conventional assays to detect the presence or absence, and/or quantity of NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex in a biological sample. Thus, in an eleventh aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8
15 NAE1ABM to a biological sample, allowing the detectable NAE1ABM to bind to NAE1-alpha, NAE1 heterodimer, or NAE1 heterodimer/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NAE1ABM and NAE1-alpha, NAE1-heterodimer, or NAE1 heterodimer/NEDD8 complex.

20 A detectable NAE1ABM is an NAE1ABM which can be detected in an assay. Such detection is preferably through the direct or indirect binding of a tag or label on the NAE1ABM. The term "direct or indirect binding" is as used previously. Preferred tags and labels include, without limitation, radioisotopes, heavy metals, fluorescent labels, chemoluminescent labels, enzymes and enzyme substrates.
25 Preferred biological samples include blood, serum, plasma, cells, tissue portions, and cell or tissue extracts. In certain preferred embodiments, the method according to this aspect of the invention takes the form of a conventional ELISA or RIA. In another preferred embodiment, the method employs either direct or indirect immunofluorescence. Additional preferred embodiments utilize *in vivo* imaging

of cells expressing NAE1-alpha using conventional imaging agents directly or indirectly bound to an NAE1ABM according to the invention.

5 Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NAE1-alpha can also be used in conventional assays to detect the presence or absence of NAE1-alpha nucleic acid in a biological sample. Thus, in a twelfth aspect, the invention provides methods for determining the presence or absence and/or quantity of NAE1-alpha nucleic acid in a biological sample. In preferred embodiments, such assays are nucleic acid
10 hybridization and/or amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically complementary to NAE1-alpha nucleic acid. Particularly preferred embodiments include Northern blotting, dot or slot blotting, and polymerase chain reaction.

15 In a thirteenth aspect, the invention provides methods for identifying modulating ligands of NAE1-alpha. Some NAE1ABMs are capable of acting as antagonists or agonists of NAE1-alpha. Thus, the method according to this aspect of the invention comprises providing NAE1ABMs to an assay system for NAE1-alpha participation in the NEDD8-activation/conjugation pathway, and determining
20 whether such NAE1ABMs interfere with or enhance the ability of NAE1-alpha to participate in the NEDD8-activation/conjugation pathway. The NAE1ABMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a mixed population of molecules, as for example in a screening procedure. This aspect of the invention includes antagonists or agonists
25 of NAE1-alpha identified by this method according to the invention. Assessment of ability to "interfere with or enhance the ability to participate in the NEDD8-activation/conjugation pathway" can conveniently be carried out using an *in vitro* activity system, as later described herein. Preferably, such interference or enhancement results in a reduction of NEDD8 activation/conjugation of at least

50%, more preferably at least 90%, and most preferably, at least 99%, or an increase of NEDD8 activation/conjugation of at least 50%, preferably at least 2-fold, more preferably at least 5-fold.

5 In a fourteenth aspect the invention provides a purified complex of NAE1-beta and NAE1-alpha, or of NAE1-beta, NAE1-alpha and NEDD8, or a purified complex of portions thereof. The term "complex" means in covalent or noncovalent association, preferably with an affinity greater than 10^6 /mole. The term "purified" is as used previously.

10 In a fifteenth aspect, the invention provides modulating ligands of NAE1-alpha. Preferred modulating ligands are NAE1ABMs which act as antagonists, interfering with the ability of NAE1-alpha to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are
15 NAE1ABMs which act as agonists, enhancing the ability of NAE1-alpha to participate in the NEDD8-activation/conjugation pathway. Preferably, such inhibition or enhancement is specific, *i.e.*, the modulating ligand interferes with or enhances the ability of NAE1-alpha to participate in the NEDD8 activation/
20 conjugation pathway at a concentration that is lower than the concentration of the ligand required to produce another, unrelated biological effect. Preferably, the concentration of the ligand required for NEDD8 activation/conjugation modulating activity is at least 2-fold lower, more preferably at least 5-fold lower, even more preferably at least 10-fold lower, and most preferably at least 20-fold lower than the concentration required to produce an unrelated biological effect. In certain
25 embodiments, such NAE1ABMs preferably interact with NAE1-alpha to inhibit or enhance the formation of NAE1 heterodimer, the formation of NEDD8 adenylate, the formation of a thiol ester bond between NEDD8 and NAE1, and/or transfer of NEDD8 to NEDD8-conjugating enzyme.

In a sixteenth aspect, the invention provides methods for modulating the conjugation of NEDD8 to NAE1 or its transfer to a NEDD8 conjugating enzyme or a target protein. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-alpha or a recombina-
5 nt expression unit which expresses NAE1-alpha or an antagonist thereof to a biological system in which NEDD8 is conjugated to a NEDD8 conjugating enzyme or a target protein.

The term "biological system", as used herein, includes *in vitro* cell or tissue extracts, cell cultures, tissue cultures, organ cultures, living plants and animals,
10 including mammals, including without limitation humans and mice. An "antagonist" is a molecule which inhibits the biological activity of NAE1.

In a seventeenth aspect, the invention provides allelic variants of NAE-1 alpha. An "allelic variant", as used herein, is a protein having at least about 75% amino acid sequence, preferably at least about 85%, more preferably at least about
15 95%, and most preferably at least about 99% identity to the amino acid sequence of NAE1-alpha, or to a portion or protein conjugate thereof which retains the biological activity of NAE1-alpha to form a heterodimer with NAE1-beta which is active in the NEDD8 activation/conjugation pathway. This aspect of the invention
20 further includes NAE1-alpha allelic variant expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid sequences encoding NAE1-alpha, or nucleic acid sequences specifically homologous or specifically complementary thereto, vectors comprising any such nucleic acid sequences, and recombinant expression units which express NAE1-beta or antisense
25 transcripts or dominant negative mutants thereof. Each of these terms is as used previously.

In a eighteenth aspect, the invention provides methods for modulating auxin response in plants. The present inventors have discovered that NAE1-alpha shares

39% identity and 61% conserved residues with Aux1 in *A. Thaliana*, which is involved in signal transduction in the auxin response in plants. This suggests that antagonists of NAE1-beta and/or NAE1-alpha should down-regulate the auxin response, and that expression of NAE1-beta and/or NAE1-alpha should up-regulate the auxin response (see Leyser *et al.*, Nature 364: 161-164 (1993)). One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-beta or NAE1-alpha or a recombinant expression unit which expresses NAE1-beta or NAE1 or an antagonist thereof to a plant that is undergoing auxin treatment.

In a nineteenth aspect, the invention provides methods for modulating the biological function of APP and/or beta peptide accumulation in a biological system. The present inventors have discovered that NAE1-alpha is substantially the same as amyloid precursor protein binding protein-1 (APP-BP1). This suggests that antagonists or agonists of NAE1-beta and/or NAE1-alpha should modulate APP function, including its role in beta peptide accumulation. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NAE1-beta or NAE1-alpha or a recombinant expression unit which expresses NAE1-beta or NAE1 or an antagonist thereof to a biological system.

In a twentieth aspect, the invention provides two new purified NEDD8-conjugating enzymes. The primary amino acid sequence of a preferred embodiment of a first such NEDD8-conjugating enzyme (NCE1) is shown in Figure 2. The primary amino acid sequence of a preferred embodiment of a second such NEDD8-conjugating enzyme (NCE2) is shown in Figure 5. However, the terms "NEDD8-conjugating enzyme 1", "NCE1", "NEDD8-conjugating enzyme 2", and "NCE2" are intended to include allelic variants thereof. An "allelic variant", as used herein, is a protein having at least about 50% amino acid sequence identity, more preferably at

least about 75%, even more preferably at least about 85%, still more preferably at least about 95%, and most preferably at least about 99% identity to the amino acid sequence set forth in SEQ ID NO 4 or SEQ ID NO 6, or to a portion or protein conjugate thereof which retains the biological activity of NCE1 or NCE2 to form a thioester linkage with NEDD8 under conditions as described in the examples below at a rate at least 10% of that of NCE1 or NCE2, preferably at least 25% as fast, more preferably at least 50% as fast, and most preferably at least 75% as fast. Preferably, such biologically active portion comprises an amino acid sequence spanning residue 111 in Figure 2 or residue 116 in Figure 5, more preferably comprises at least about 25 additional amino acids of respectively NCE1 or NCE2, even more preferably at least about 50 additional amino acids of respectively NCE1 or NCE2, still more preferably at least about 75 additional amino acids of respectively NCE1 or NCE2, yet even more preferably at least about 100 additional amino acids of respectively NCE1 or NCE2, most preferably at least about 150 additional amino acids from respectively NCE1 or NCE2. Such allelic variants have the biological activity of NCE1 or NCE2, as discussed above. In alternative preferred embodiments, such allelic variants are either rationally designed or naturally occurring allelic variants, *i.e.*, they are expressed in actual individual mammals, most preferably from actual individual humans or mice. Rationally designed allelic variants can be produced according to standard art-recognized procedures (see *e.g.*, international publication WO95/18974). The terms "purified" and "protein" are as used previously.

In a twenty-first aspect, the invention provides NEDD8-conjugation enzyme expression elements. Such elements include, without limitation, isolated or recombinant nucleic acid sequences encoding NCE1 or NCE2 or dominant negative mutants thereof, or capable of expressing antisense transcripts thereof or nucleic acid sequences specifically homologous or specifically complementary thereto, and vectors comprising any such recombinant expression elements, preferably expression vectors.

The terms "specifically homologous", "specifically complementary" and "specifically hybridizes" are as used previously. A "recombinant expression element" is a nucleic acid sequence which encodes NCE1 or NCE2, or a portion encoding at least 20 contiguous amino acids thereof, or a dominant negative mutant thereof, or is capable of expressing an antisense molecule specifically complementary thereto, or a sense molecule specifically homologous thereto wherein the recombinant expression unit may be in the form of linear DNA or RNA, covalently closed circular DNA or RNA, or as part of a chromosome, provided however that it cannot be the native chromosomal locus for NCE1 or NCE2. Preferred recombinant expression elements are vectors, which may include an origin of replication and are thus replicatable in one or more cell type. Certain preferred recombinant expression elements are expression vectors, and further comprise at least a promoter and passive terminator, thereby allowing transcription of the recombinant expression element in a bacterial, fungal, plant, insect or mammalian cell. Preferred recombinant expression elements have at least 75% nucleic acid sequence identity with the nucleic acid sequence set forth in SEQ ID NO 2 OR SEQ ID NO 4, more preferably at least 90%, even more preferably at least 95%, and most preferably at least 99%, and encode a protein or peptide having either NCE1 or NCE2 biological activity or activity as a dominant negative mutant thereof, as further described below.

"Dominant negative mutants" are proteins or peptides derived from NCE1 or NCE2 which inhibit the biological activity of, respectively NCE1 or NCE2. Preferred dominant negative mutants include variants in which the C at position 111 of NCE1 or position 116 of NCE2 is substituted, preferably by S. Preferred dominant negative mutants interfere with association of NEDD8 and NCE1 or NCE2 and can be derived from, respectively, NCE1 or NCE2. Other preferred dominant negative mutants interfere with conjugation of NEDD8 to a target protein and can be derived from either NCE1 or NCE2. Such dominant negative mutants can be prepared by art recognized procedures (see *e.g.*, Townsley *et al.*, Proc. Natl.

Acad. Sci. USA 94: 2362-2367 (1997)). Preferably, such dominant negative mutant is a protein or peptide having from 50% amino acid sequence identity to about 99% identity to the amino acid sequence set forth in SEQ ID NO 3 or SEQ ID NO 5, or to a portion or protein conjugate thereof which inhibits the biological activity of NCE1 or NCE2 to form a thioester linkage with NEDD8 under conditions as described in the following examples by at least 50%, preferably by at least 75%, more preferably by at least 90% and most preferably by at least 99%. Preferably, such inhibitory portion comprises an amino acid sequence spanning residue 111 in Figure 2 or residue 116 in Figure 5, more preferably comprises at least about 25 additional amino acids of respectively NCE1 or NCE2, even more preferably at least about 50 additional amino acids of respectively NCE1 or NCE2, still more preferably at least about 75 additional amino acids of respectively NCE1 or NCE2, yet even more preferably at least about 100 additional amino acids of respectively NCE1 or NCE2, most preferably at least about 150 additional amino acids from respectively NCE1 or NCE2.

The purified protein and its structural information provided herein enables the preparation of NCE1 and NCE2 binding molecules, respectively NCE1BMs and NCE2BMs. Thus, in a twenty second aspect, the invention provides methods for identifying NCE1BMs and NCE2BMs. One preferred method according to this aspect of the invention comprises screening for NCE1BMs or NCE2BMs by contacting purified NCE1 or NCE2 according to the invention and populations of molecules or mixed populations of molecules and determining the presence of molecules which bind specifically to NCE1 or NCE2. Another preferred method according to this aspect of the invention comprises rationally designing molecules to bind NCE1 or NCE2 based upon structural information from the purified NCE1 or NCE2 provided by the invention and determining whether such rationally designed molecules bind specifically to NCE1 or NCE2. Molecules that bind specifically to NCE1 or NCE2 are molecules that bind to NCE1 or NCE2 with greater affinity than to other unrelated proteins. Preferably, binding affinity of the molecule is at least 5-fold greater than

affinity for unrelated proteins, more preferably at least 10-fold greater, still more preferably at least 50-fold greater, and most preferably at least 100-fold greater. This aspect of the invention includes NCE1BMs or NCE2BMs identified by the methods according to the invention.

5 As used herein, a "NCE1 or NCE2 -binding molecule", or "NCE1BM or NCE2BM", is a molecule or macromolecule which binds under physiological conditions to, respectively NCE1 or NCE2. The terms "binds under physiological conditions", "population of molecules" and "mixed population of molecules" are as used previously.

10 In certain preferred embodiments, an NCE1BM or NCE2BM according to the invention is a peptide or a peptidomimetic. For purposes of the invention, the term "peptide" is as used previously.

15 In certain preferred embodiments, such an NCE1BM or NCE2BM comprises a complementarity determining region of an antibody which binds under physiological conditions to a peptide-containing epitope of, respectively NCE1 or NCE2, or a peptidomimetic of such a complementarity determining region. For purposes of the invention, the term "complementarity determining region of an antibody" is as used previously. Accordingly, all such antibody derivatives and peptidomimetics thereof are contemplated to be within the scope of the present
20 invention. Compositions according to the invention may further include physiologically acceptable diluents, stabilizing agents, localizing agents or buffers.

Additional preferred NCE1BMs and NCE2BMs according to the invention include small molecules, which can be identified using screening or rational design approaches as discussed later herein.

25 NCE1BMs and NCE2BMs can be used in conventional assays to detect the presence or absence, and/or quantity of NCE1, or NCE2, or NCE1 or NCE2/NEDD8 complex in a biological sample. Thus, in a twenty-third aspect, the invention provides methods for determining the presence or absence and/or quantity of NCE1

or NCE2, or NCE1 or NCE2/NEDD8 complex in a biological sample. Such methods comprise providing a detectable NCE1BM or NCE2BM to a biological sample, allowing the detectable NCE1BM or NCE2BM to bind to NCE1, or NCE1 or NCE2/NEDD8 complex, if any is present in the biological sample, and detecting the presence or absence and/or quantity of a complex of the detectable NCE1BM or NCE2BM and, respectively, NCE1 or NCE2, or NCE1 or NCE2/NEDD8 complex.

A detectable NCE1BM or NCE2BM is an NCE1BM or NCE2BM which can be detected in an assay. Such detection is preferably through the direct or indirect binding of a tag or label on the NCE1BM or NCE2BM. The term "direct or indirect binding" is as used previously. Preferred tags and labels include, without limitation, radioisotopes, heavy metals, fluorescent labels, chemoluminescent labels, enzymes and enzyme substrates. Preferred biological samples include blood, serum, plasma, cells, tissue portions, and cell or tissue extracts. In certain preferred embodiments, the method according to this aspect of the invention takes the form of a conventional ELISA or RIA. In another preferred embodiment, the method employs either direct or indirect immunofluorescence. Additional preferred embodiments utilize *in vivo* imaging of cells expressing NCE1 or NCE2 using conventional imaging agents directly or indirectly bound to an NCE1BM or NCE2BM according to the invention.

Nucleic acid sequences specifically complementary to and/or specifically homologous to nucleic acid sequences encoding NCE1 or NCE2 can also be used in conventional assays to detect the presence or absence of NCE1 or NCE2 nucleic acid in a biological sample. Thus, in a twenty-fourth aspect, the invention provides methods for determining the presence or absence and/or quantity of NCE1 or NCE2 nucleic acid in a biological sample. In preferred embodiments, such assays are nucleic acid hybridization and/or amplification assays, such assays comprising providing to the biological sample a nucleic acid sequence which is specifically complementary to NCE1 or NCE2 nucleic acid. Particularly preferred embodiments

include Northern blotting, dot or slot blotting, and polymerase chain reaction.

5 In a twenty-fifth aspect, the invention provides methods for identifying modulating ligands of NCE1 or NCE2. Some NCE1BMs and NCE2BMs are capable of acting as antagonists or agonists of, respectively, NCE1 and NCE2. Thus, the method according to this aspect of the invention comprises providing NCE1BMs or NCE2BMs to an assay system for, respectively, NCE1 or NCE2 participation in the NEDD8-activation/conjugation pathway, and determining whether such NCE1BMs or NCE2BMs interfere with or enhance the ability of NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. The NCE1BMs or NCE2BMs are preferably provided as a population of molecules (most preferably rationally designed molecules), or as a mixed population of molecules, as for example in a screening procedure. This aspect of the invention includes antagonists or agonists of NCE1 or NCE2 identified by this method according to the invention. Assessment of ability to "interfere with or enhance the ability to participate in the NEDD8-activation/conjugation pathway" can conveniently be carried out using an *in vitro* activity system, as later described herein. Preferably, such interference or enhancement results in a reduction of NEDD8 activation/conjugation of at least 50%, more preferably at least 90%, and most preferably, at least 99%, or an increase of NEDD8 activation/conjugation of at least 50%, preferably at least 2-fold, more preferably at least 5-fold, most preferably at least 10-fold.

25 In a twenty-sixth aspect, the invention provides modulating ligands of NCE1 or NCE2. Preferred modulating ligands are NCE1BMs or NCE2BMs which act as antagonists, interfering with the ability of, respectively, NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. Other preferred modulating ligands are NCE1BMs or NCE2BMs which act as agonists, enhancing the ability of, respectively NCE1 or NCE2 to participate in the NEDD8-activation/conjugation pathway. Preferably, such inhibition or enhancement is specific, *i.e.*, the modulating

ligand interferes with or enhances the ability of NCE1 or NCE2 to participate in the NEDD8 activation/ conjugation pathway at a concentration that is lower than the concentration of the ligand required to produce another, unrelated biological effect. Preferably, the concentration of the ligand required for NEDD8

5 activation/conjugation modulating activity is at least 2-fold lower, more preferably at least 5-fold lower, even more preferably at least 10-fold lower, and most preferably at least 20-fold lower than the concentration required to produce an unrelated biological effect. In certain embodiments, such NCE1BMs or NCE2BMs preferably interact with, respectively, NCE1 or NCE2 to inhibit or enhance the formation of a
10 thiol ester bond between NEDD8 and NCE1 or NCE2, and/or transfer of NEDD8 to a target protein.

In a twenty-seventh aspect, the invention provides methods for modulating the formation of a thiol ester bond between NEDD8 and NCE1 or NCE2, or transfer
15 of NEDD8 to a target protein. One preferred embodiment of the method according to this aspect of the invention comprises providing a modulating ligand of NCE1 or NCE2 or a recombinant expression unit which expresses NCE1 or NCE2 or an antagonist thereof to a biological system in which NEDD8 is conjugated to another protein. The term "biological system", as used herein, includes *in vitro* cell or tissue
20 extracts, cell cultures, tissue cultures, organ cultures, living plants and animals, including mammals, including without limitation humans and mice.

In a twenty-eighth aspect, the invention provides oligonucleotides that are specifically complementary to a portion of a nucleotide sequence shown in Figure 2
25 or Figure 5. For purposes of the invention, the term "oligonucleotide" is as used previously. Certain embodiments of such oligonucleotides are useful as antisense probes. Other embodiments are useful as antisense oligonucleotides for use in animal model or human therapeutic settings.

In a twenty-ninth aspect the invention provides a purified complex of NCE1 and NEDD8, or of NCE2 and NEDD8. The terms "complex" and "purified" are as used previously.

5

The following examples are intended to further illustrate certain particularly preferred embodiments of the invention and are not intended to limit the scope of the invention. Searches of the human EST database utilized the program BLAST (Altschul *et al.*, Nucleic Acids Res 25: 3389-3402 (1997)). Searches for transmembrane helices used the program Antheptrot V.3.0 Gilbert Deleague, Institute de Biologie et Chemie des Proteines 69 367 Lyon cdex 07, France.

10

Example 1

Preparation of Human NEDD8

15

Nucleotide sequence coding the N-terminal 76 residues of human Nedd8 was obtained from a human leukocyte cDNA Library (Life Technologies Tech-LineSM, Inc) by nested polymerase chain reaction, using 5'-ccg tgt gca gcc cca aac tgg and 5'-aca ggg taa aga ggt aaa atg as the first round forward and reverse primer, respectively. In the second round, 5'-ggg aat tcc ata tgc taa tta aag tga aga cgc and 5'-ccc aag ctt tca tcc tcc tct cag agc caa cac were used as the forward and reverse primer, respectively. The second PCR product was digested with NdeI and HindIII and ligated to the large fragment of a similarly digested PT7-7 vector. The construct was transformed into the E.coli strain BL21(DE3)/pLysS (Novagen). Nedd8 expression was induced by the addition of 0.5 mM IPTG. The S100 fraction of bacterial extracts was applied to a Q-Sepharose column in 50 mM HEPES, pH 7.5 and the flow-through which contained Nedd8 was collected, concentrated by ultrafiltration and fractionated by size exclusion chromatography on Superdex G75.

20

25

Example 2

Identification of NEDD8-Activating Enzyme

To identify the human Nedd8-activating enzyme, we first tested for the presence of this enzyme activity by monitoring the incorporation of Nedd8 in the form of a thioester linkage into proteins derived from Hela cells. On the basis of the chromatographic behavior of recombinant human Nedd8, we generated from Hela cell extracts two protein fractions (FI and FII) which are expected to be devoid of endogenous Nedd8 as follows. To remove Nedd8, 400 mg of protein from Hela cell S100 fraction was applied to a 70 ml Q-Sepharose column, equilibrated with 50 mM HEPES, pH 8.0 with 1 mM DTT. Proteins in the flow-through fraction were precipitated in 90% ammonium sulfate, dialyzed and fractionated by size exclusion chromatography on Superdex G75. Fractions which eluted earlier than Nedd8 were pooled and concentrated by ultrafiltration to 15mg/ml and is designated here as FI. Proteins retained by the Q-Sepharose were eluted by inclusion of 0.6 M NaCl in the equilibration buffer. The collected proteins were precipitated with 90% ammonium sulfate and dialyzed against 25 mM Hepes, pH7.5, and 1 mM DTT and concentrated to 15 mg/ml of protein. This fraction is designated here as FII. Fraction II was generated by collecting proteins that were retained by an anion-exchange gel (Q-Sepharose) while FI was obtained by further fractionation of unretained proteins by gel filtration. Incubation of ^{125}I -Nedd8 with FII, but not with FI, produced a radiolabeled band on SDS-gel which migrated at 59 kDa. Formation of this radiolabeled species required the presence of ATP, and this species could not be detected when DTT was included in the SDS-gel sample buffer. Thus, FII contains an activity which attaches Nedd8 to a protein via a DTT-sensitive linkage. Incubation of ^{125}I -Nedd8 with FI and FII together resulted in the formation of two additional radiolabeled bands on SDS-gel, migrating at 30 and 97 kDa. Only the 30 kDa species exhibited DTT sensitivity. One interpretation of this result is the presence of a Nedd8-conjugating enzyme in FI which serves to accept Nedd8 from its activating

enzyme in FII to form a 30 kDa thioester.

Example 3

Purification of NEDD8-Activating Enzyme

5 To purify the protein in FII which forms the DTT-sensitive linkage to Nedd8, we immobilized Nedd8 to CH-Sepharose 4B gels and used DTT to elute proteins that were initially retained by the gel matrix, as follows. Nedd8-affinity gel was prepared by coupling purified Nedd8 to activated CH Sepharose 4B (Pharmacia) according to
10 manufacturer's instructions and lead to the coupling of 5 mg of Nedd8/ml of gel beads. 100 mg of FII protein in a 9 ml reaction buffer containing MgATP and an ATP regenerating system was applied to 1 ml of Nedd8-immobilized gel beads at room temperature. The column was washed sequentially with 5 bed volumes of buffer A (50 mM Tris-HCl buffer, pH 7.5), buffer A with 0.5M NaCl, and buffer A. A buffer
15 containing 50 mM Tris-HCl, pH 9.0 and 10 mM DTT was used to elute bound proteins. Analysis of the eluted proteins by SDS-PAGE and silver-staining revealed the presence of two major proteins that migrated at 60 and 49 kDa. A third major protein, migrating at 43 kDa, eluted as a broad peak. When the eluted proteins were analyzed by gel filtration chromatography, the 43 kDa protein eluted as a large
20 aggregate at the void volume while p60 and p49 were found to co-elute with a retention time similar to that of the 110 kDa ubiquitin-activating enzyme, suggesting that these two proteins form a heterodimer. To determine which one of these two proteins forms the DTT-sensitive linkage with Nedd8, proteins purified from the Nedd8-affinity chromatography step were tested. The result is consistent with p49 being the Nedd8 acceptor. This protein is quantitatively absent only when
25 ATP or AMPPNP was included in the reaction and only if the electrophoresis was carried out in the absence of DTT. The fact that no new discrete protein band was detected under conditions in which p49 was absent is likely due to the presence of p60 which precludes the detection of proteins that would migrate with similar mobility. In a separate experiment, the use of ¹²⁵I-Nedd8 in the reaction led to the

detection of a DTT-sensitive 59 kDa band, confirming the presence of a Nedd8-containing thioester. The ability of AMPPNP to substitute for ATP suggests that NEDD8 activation, similar to ubiquitin and SUMO-1, involves the intermediate formation of an enzyme-bound Nedd8-adenylate prior to thioester linkage.

Example 4

Sequence Determination of NEDD8-Activating Enzyme

To obtain the identity of p49, this protein was excised from an SDS-gel, digested with trypsin and peptides were eluted and purified by HPLC as follows. The peak fractions from Nedd8-affinity chromatography step were concentrated and separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and bands corresponding to p49 and p60 were excised. The gel slices were digested with trypsin, peptides were extracted and purified by microbore reversed-phase HPLC (PE-Applied Biosystems model 140A/1000S system) on Zorbax SB-C18 silica columns (1x150 mm), using linear gradients of acetonitrile in 0.08% aqueous trifluoroacetic acid (TFA), essentially as described in (J. Pohl et al, FEBS Lett. 272, 200, 1990.). The masses of the peptides were determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF) using a Bruker Instruments model ProFlex MALDI-TOF instrument operated in the reflectron mode; 2,5-dihydroxybenzoic acid was used as the sample matrix. The sequences of the peptides were determined by automated Edman degradation on a PE-Applied Biosystems model Procise-HT sequencer system operated in the pulsed-liquid mode using manufacturer's supplied sequencing cycles. Two tryptic peptide sequences were determined (shown as underlined in Figure 1), and these sequences were used to search the protein as well as the expressed sequence tag (EST) data bases. Although these sequences did not match known proteins in the data bases, two groups of EST clones could be identified whose translated amino acid sequence yielded perfect matches to either one of the two tryptic peptides. Further homology search with these EST sequences identified additional EST clones with overlapping sequences. Analysis of these EST

clones enabled us to obtain a contiguous open reading frame (ORF) that encodes a 442-residue protein which contains the two tryptic peptide sequences. The nucleotide sequence of this ORF was confirmed by direct nucleotide sequencing of two EST clones (AA40862 and R57021). Analysis of this protein sequence revealed three regions of homology with human Uba1. Region I contains the putative ATP binding site found in Uba1 which is also present in yeast Uba2, and region II contains the PXCT sequence motif found in Uba1 in which the cysteine residue was identified by mutational analysis to form thiolester linkage with ubiquitin. These similarities are expected if the activation of Nedd8 utilizes a mechanism similar to that of ubiquitin and Smt3. Since p49 forms a heterodimer with p60 and functions as a protein component of Nedd8 activation, we designate it as Nae._{beta} and p60 as Nae._{alpha}. Searches of the data banks with this protein sequence identified an open reading frame in *S. pombe*, and one in *C. elegans* which code for similar size proteins. In addition, a *S. cerevisiae* 299-residue protein, despite its smaller size, also shows extensive homology with this human protein. These are likely homologues of Nae._{beta} in different species since identical and highly conserved residues among these four proteins are interspersed throughout most of the protein whereas their homology to Uba1 and Uba2 is limited to defined regions only.

Example 5

Identification of Nae-alpha

The similarity between Nedd8- and Smt3-activating enzyme in their subunit structure suggested that p60 or Nae._{alpha} would also contain a sequence stretch that shares homology to the N-terminal portion of Uba1. Using procedures similar to those with p49, three tryptic peptide sequences were obtained for p60. These sequences FTVVATQLPEXTXL, EHFQSYDLDHME, and QTPSEFWILA yielded perfect matches to residues 123-138, 194-205 and 300-308 in the 534-residue APP-BP1. In

addition mass spectrometry of 15 of the tryptic peptides revealed matches within 1 Da of the expected mass of tryptic peptides of APP-BP1. These matches covered 37% of the APP-BP1 sequence. Thus, we concluded that Nae- α is indeed APP-BP1.

5

Example 6

Identification and cloning of NCE1

The putative human homolog of yeast Ubc12 was identified by searching the human EST database for clones having coding sequences that are homologous to the yeast protein. An initial search using the yeast protein sequence identified several clones. Clone AA261836, which contains a coding sequence very similar to a region of the yeast protein was used to search for further EST clones. The search led to the construction of a contiguous consensus sequence from overlapping clones which predicts a gene to encode a protein having 183 amino acids, with a predicted molecular mass of 20899 Da. The contiguous nucleotide sequence was obtained using nested PCR on a human leukocyte cDNA library. The first PCR used primers having the sequence GCAGGATGATCAAGCTGTTCTCGC (forward) and CGTGCGCGGGGTGGGTATGCGCCA (reversed). The second PCR used the primers CGGGAATTCCATATGATCAAGCTGTTCTCGCTG (forward) and CGCCCAAGCTTCTATTTCAAGCAGCGCTCAAAG (reversed). The PCR product was digested with NdeI and HindIII and ligated with similarly digested plasmid pT7-7. The resulting clone, pT7-7-UbcH12, was sequenced to determine the nucleotide sequence [SEQ ID NO 3] and deduced amino acid sequence [SEQ ID NO 4] shown in Figure 1. Figure 2 shows the alignment of NCE1 with yeast Ubc12. NCE1 shows 41% identity and 63% homology with yeast Ubc12.

Example 7

Expression and purification of NCE1

BL21 (DE3) bacterial cells (Novagen, Madison, WI; catalog no. 69450-1) were transformed with pT7-7-UbcH12 plasmid using conventional procedures. The transformed bacteria were induced to express the NCE1 protein by adding, to a final concentration of 1 mM, isopropyl-b-D-thiogalactopyranoside (IPTG) to an exponentially growing culture. The culture was allowed to grow for an additional 3 hours at 37°C. NCE1 protein was purified from lysed cells by sequential anion exchange and size exclusion chromatography. For anion exchange chromatography, the bacterial extract was loaded at a protein/gel ratio of 15 mg protein/ml gel onto Q-Sepharose (Pharmacia, Piscataway, NJ) equilibrated with 50 mM HEPES (pH 7.8) and 1mM DTT. NCE1 protein was retained by the gel and eluted using a linear NaCl gradient in the gel equilibration buffer. Fractions containing NCE1 protein were determined by assaying for NEDD8 thioester formation. NCE1 was found to elute at 0.08 M NaCl. Active fractions were pooled and concentrated by microfiltration and then subjected to size exclusion chromatography on Superdex-75 (Pharmacia) using a column buffer of 50 mM HEPES (pH 7.8), 1 mM DTT and 50 mM NaCl. Fractions were assayed for NEDD8-thioester formation. NCE1 eluted at a volume expected for a 19kDa protein, suggesting that it exists as a monomer. SDS-PAGE analysis with Coomassie stain indicated that the preparation was predominantly (>90%) NCE1 protein. Purified NCE1 protein migrated on an 8% TRICINE gel at a molecular weight of 21 kDa (data not shown). Extending the N-terminus of NCE1 with the amino acid sequence MHHHHHHH resulted in an NCE1 variant protein that retained activity in NEDD8-thioester formation. The six histidine residues provide a nickel binding site and allowed this variant to be purified with Ni-NTA or other metal affinity chromatography procedures.

Example 8

Thioester formation between NCE1 and NEDD8

Proteins (as indicated below) were incubated in a reaction buffer containing 25 mM Hepes (pH7.0), 10 mM Mg^{2+} and 1 mM ATP for 5 minutes at 30°C. The reaction was stopped by addition of SDS sample loading buffer. Each sample was divided into two aliquots, to one of which was added DTT to a final concentration of 10 mM. The DTT-containing sample was heated in a 95°C bath for two minutes. Samples were separated on 10% SDS-Tricine PAGE, followed by silver staining. The results are shown in Figure 4. Lanes 1-4 are reaction mixtures 1-4. Lanes 5-8 are reaction mixtures 1-4 which were incubated with 10 mM DTT and heated to 90°C for two minutes prior to electrophoresis. These results show that NCE1 migrates at a slower rate in the presence of NEDD8 and Nae, and that this is reversible by DTT. Ubiquitin activating enzyme, E1, cannot substitute for NAE in providing this result. These data support the view that NCE1 is a NEDD8 conjugating enzyme which forms a thioester with NEDD8 in the presence of activating enzyme, NAE.

<u>Reaction No.</u>	<u>Proteins</u>
1	NAE + NEDD8
2	NCE1 + NEDD8
3	NCE1 + NEDD8 + NAE
4	NCE1 + NEDD8 + ubiquitin activating enzyme, E1

Example 9

Identification and cloning of NCE2

The human EST database was searched using as query sequence HPNITETICLSLLREHSIDGTGWA. This is the sequence of clone AA306113 and bears similarity to the active site of proteins in the UBC protein family. Clones were identified which had sequences overlapping the sequence of clone AA306113. The identified sequences of the overlapping EST clones were aligned by the program

CLUSTALW (See Thompson *et al.*, Nucleic Acids Res. 22: 4673-4680 (1994), or by the program SeqMan (DNASTAR, Inc., Madison, WI) to yield a consensus sequence, CON1. CON 1 was used to perform searches for additional clones with overlapping sequences. The overlapping sequences yielded an open reading frame which
5 encodes a protein of 185 amino acids (predicted molecular mass = 21076 Da). Based upon homology to known human Ubc proteins, this gene is a member of the human Ubc gene family. The contiguous nucleotide sequence of NCE2 was obtained using nested PCR on a human leukocyte cDNA library. The first PCR used the primers AGCCCAGGGTAAAGGCAGCA (forward) and
10 CATGTTAGAGACAAACTGTA (reversed). The second PCR used the primers GGGAATTCCATATGCTAACGCTAGCAAGTAA (forward) and CCATCGATTTCATCTGGCATAACGTTTGA (reversed). The PCR product was then cloned into the NdeI/HindIII sites of pT7-7 to generate the plasmid pT7-7-HSUBC17. The sequence of the NCE2 gene and its deduced amino acid sequence are shown in
15 Figure 4. No close homolog exists in the yeast genome. The protein has 46% identity and 64% homology with a *C. elegans* gene (Genebank Accession # CE275850) of unknown function (see figure 5).

Example 10

20 Expression and purification of NCE2

BL21 (DE3) bacterial cells were transformed with pT7-7-UbcH17 plasmid using conventional procedures. The transformed bacteria were induced to express the NCE2 protein by adding, to a final concentration of 1 mM, isopropyl-b-D-thiogalactopyranoside (IPTG) to an exponentially growing culture. The culture was
25 allowed to grow for an additional 3 hours at 37°C. NCE2 protein was purified from lysed cells by sequential anion exchange and size exclusion chromatography. For anion exchange chromatography, the bacterial extract was loaded at a protein/gel ratio of 15 mg protein/ml gel onto Q-Sepharose (Pharmacia) equilibrated with 50 mM HEPES (pH 7.8) and 1mM DTT. NCE2 protein was retained by the gel and

eluted using a linear NaCl gradient in the gel equilibration buffer. Fractions containing NCE2 protein were determined by assaying for NEDD8 thioester formation. NCE2 was found to elute at 0.8 M NaCl. Active fractions were pooled and concentrated by microfiltration and then subjected to size exclusion chromatography on Superdex-75 (Pharmacia) using a column buffer of 50 mM HEPES (pH 7.8), 1 mM DTT and 50 mM NaCl. Fractions were assayed for ¹²⁵I-NEDD8-thioester formation. NCE2 eluted at a volume expected for a 21 kDa protein, suggesting that it exists as a monomer. SDS-PAGE analysis with Coomassie stain indicated that the preparation was predominantly (>90%) NCE2 protein. Purified NCE2 protein migrated on an 8% TRICINE gel at a molecular weight of 21 kDa (data not shown).

Example 11

Thioester formation between NCE2 and NEDD8

The ability of NCE2 to form a thioester bond with NEDD8 was assessed as follows. NCE2 protein, either purified or from bacterial lysate, was incubated with ¹²⁵I-NEDD8 (10⁶ cpm/μg) in a buffer containing 25 mM HEPES (pH 7.0), 10 mM MgCl₂, 1 mM ATP and 20nM purified NAE1 or ubiquitin-activating enzyme. The reaction was allowed to proceed at 30°C for 5 minutes. The reaction was stopped by adding SDS-sample buffer either with or without 10 mM DTT. The samples were subjected to SDS-PAGE and autoradiography. In the reaction containing NCE2 (lane 3), the autoradiograph showed two radiolabeled bands with apparent molecular masses of 7 and 29 kDa, which are the expected molecular masses of NEDD8 and NEDD8-NCE2, respectively. Only the 7kDa band was detected when the sample was incubated in 10 mM DTT prior to electrophoresis, consistent with the 29 kDa band being a NEDD8-NCE2 thioester. Analogous reactions containing NCE1 in place of NCE2 (lanes 2 and 4) are shown for comparison. These results demonstrate that NCE2 is capable of forming a thioester bond with NEDD8, but not with ubiquitin, in

a NAE-dependent reaction. These data support the view that NCE2 is a NEDD8 conjugating enzyme.

Example 12

5 Preparation of dominant negative mutants

 The active site cysteine of a cloned NCE1 or NCE2 is assigned by examining the sequence alignment with known Ubc proteins (see Figure 6 for alignment). The active site cysteine is replaced by a serine using standard site-specific mutagenesis. The mutant protein is expressed in bacteria and purified. The ability of the mutant
10 protein to form a stable oxygen ester with NEDD8 is established as described in Examples 8 and 11 above, except that the bond formation is not labile in DTT. Dominant negative mutant activity is then established by introducing the mutant protein in increasing concentrations in an assay as described in Examples 8 and 11 above and demonstrating dose-dependent inhibition of NEDD8/NCE1 or NCE2
15 complex formation.